Pestic. Sci. 1997, **50**, 28–34

Alternative Respiration: a Biochemical Mechanism of Resistance to Azoxystrobin (ICIA 5504) in *Septoria tritici*

Basil N. Ziogas,[a]* Brian C. Baldwin[b] & Jane E. Young[b]

[a] Laboratory of Plant Pathology, Agricultural University of Athens, Votanikos, 118 55 Athens, Greece
[b] Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire RG42 6ET, UK

(Received 1 July 1996; revised version received 18 October 1996; accepted 21 January 1997)

Abstract: The mechanism of resistance to ICIA 5504 (azoxystrobin) in a *Septoria tritici* mutant raised in the laboratory has been investigated. This mutant was approximately 10 times less sensitive than the wild-type strain in in-vitro tests towards spore germination or fungal growth. Glucose oxidation in whole cells was inhibited in the wild type (80% inhibition at 0·1 μg ml^{-1}), whereas in the resistant mutant, oxygen uptake was stimulated (50% stimulation at 1·0 μg ml^{-1}). Respiration of the wild-type strain was inhibited by antimycin A and cyanide but not that of the mutant. These results indicate the existence of an efficient alternative respiratory pathway in the mutant, which was inhibited by the addition of 2 mM salicylhydroxamate (SHAM). Using mitochondria, antimycin A and ICIA 5504 did not completely inhibit NADH oxidation in either strain. Addition of SHAM inhibited part of the antimycin- and ICIA 5504-insensitive oxygen uptake only in mutant mitochondria. For complete inhibition of oxygen reduction, SHAM and cyanide need to be present. Thus, three systems of electron transfer from exogenous NADH to oxygen are present in *S. tritici* mitochondria: the cytochrome pathway which is sensitive to ICIA 5504 and antimycin A inhibition in both strains, the system of NADH-cytochrome *c* reductase which bypasses the methoxyacrylate inhibition at the cytochrome bc_1 complex, and the alternative oxidase which is inhibited by SHAM, and which is partially functioning only in mitochondria isolated from the ICIA 5504-resistant mutant.

When the *S. tritici* isolates were tested for their in-vivo sensitivity to ICIA 5504 on wheat, the resistant strain was controlled better than the wild type. This indicates that the decreased ATP formation by the alternative pathway of respiration was inadequate for efficient parasitic growth on the host.

Key words: fungicide, β-methoxyacrylate, strobilurins, electron transport, respiration, alternative pathway, resistance

1 INTRODUCTION

The natural products oudemansins, strobilurins and myxothiazols, which have been shown to act at the same site in the respiratory chain at the level of cytochrome bc_1 complex,[1] have given rise to a novel type of agricultural fungicides, the β-methoxyacrylates (or strobilurins).[2] Azoxystrobin (ICIA 5504; Fig. 1) is a novel broad-spectrum fungicide of this class with a high level of activity in a wide range of crops.[3,4] The mode of action has been confirmed to be in mitochondrial electron transport, at the Q_0 centre of cytochrome bc_1 (complex III).[5–7]

A serious question for any new compound considered for development is the duration of its effectiveness against the target pathogens and whether field resistance will arise quickly. Defining the resistance risk is

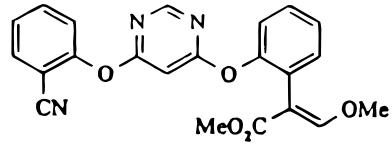

Fig. 1. Structure of methoxyacrylate fungicide ICIA 5504.

* To whom correspondence should be addressed.

Pestic. Sci. 0031-613X/97/$17.50

not easy, and one important consideration is the possible mechanism(s) by which resistance is achieved in the pathogen population. We therefore undertook this study to examine the biochemical basis of resistance in an isolate of *Septoria tritici* Rob. ex Desm. which had been produced in the laboratories of Zeneca Agrochemicals.

2 EXPERIMENTAL METHODS

2.1 Strains and culturing conditions

The wild-type strain K1097 and a methoxyacrylate-resistant isolate K1775 of *S. tritici* were used in this study. The mutant had been obtained from the wild-type strain following ultraviolet irradiation and selection on medium containing ICIA 5504 by S. P. Heaney of Zeneca Agrochemicals.

Sporidia were obtained by growing the fungus in a liquid medium containing glucose (15 g litre^{-1}), peptone (7 g litre^{-1}) and yeast extract (1·4 g litre^{-1}). Cultures were incubated at 20°C on a rotary shaker at 120 rev. min^{-1}. Under these conditions the fungus presents a yeast-like growth by sporidial budding. Strains were maintained on agar plates at 20°C containing the above medium with agar (15 g litre^{-1}).

2.2 Chemicals

ICIA 5504 (methyl (*E*)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate) was technical grade. Antimycin A and salicylhydroxamic acid (SHAM) were purchased from Sigma Chemical Company and potassium cyanide from Merck. Peptone and yeast extract were from Difco. All other chemicals, not specifically mentioned here, were reagent grade from either Sigma or BDH. Stock solutions of ICIA 5504, antimycin A and SHAM were prepared in methanol and of potassium cyanide in water. Methanol did not exceed 10 ml litre^{-1} in culture medium or reaction mixtures.

2.3 Measurement of fungitoxicity

The fungitoxicity of ICIA 5504 was measured in agar and liquid media. In the first case the sporidia were plated on agar medium with and without fungicide. The proportion of sporidia capable of producing germ tubes was counted after two days of incubation at 20°C. In the liquid medium, the effect of the toxicant on growth was determined by measuring optical density (OD) changes (at 450 nm) of sporidial suspensions. The fungicide was added to the medium in methanol and the medium was inoculated with appropriate actively growing sporidia initially to an OD of 0·3 (10^6 sporidia ml^{-1}). The cultures were shaken at 20°C and the OD changes were recorded after 48 h incubation.

2.4 Assessment of disease control on wheat

Wheat seeds (*Triticum aestivum* L., cv. Hornet) were sown (five per pot) in John Innes No. 2 compost in 8-cm pots and maintained in a growth room (16 h under fluorescent lighting at 21°C and 60% relative humidity, followed by 8 h dark at 17°C and 100% humidity). After seven days the seedlings (five pots per treatment) were sprayed with the appropriate ICIA 5504 concentrations made up in aqueous 'Tween' 20 (0·5 g litre^{-1}) with a hand-held De Vilbiss spray gun to maximum retention of droplets. The spray was allowed to dry and, after 2 h, the seedlings were inoculated with a sporidial suspension of the *S. tritici* isolate, prepared from Petri dish cultures grown on potato dextrose agar, diluted to 10^6 sporidia ml^{-1}, using the spray gun, to maximum retention. The inoculation was repeated after the droplets had dried, and the plants were placed in a humidity chamber (100% humidity at 20°C for 48 h), before returning them to the growth room. Uninoculated control plants were sprayed with the 'Tween' 20 solution alone. In the 2-h protectant disease test, the percentage disease cover on the first fully expanded leaf was assessed relative to untreated inoculated plants after four weeks.

2.5 Measurement of sporidial substrate oxidation

For whole-cell respiration studies, 48-h sporidia grown in liquid medium were suspended in a respiration medium containing glucose (5 g litre^{-1}), magnesium sulfate (2 mM) and yeast extract (5 g litre^{-1}). The sporidial concentration was adjusted to 5·5 mg dry weight ml^{-1} (equivalent to an OD of 6·3–6·5) and the suspension was shaken at 20°C. The inhibitors were added in methanol (ICIA 5504, antimycin A and SHAM) or in water (potassium cyanide). After 1 h incubation, samples (2·5 ml) were withdrawn and the rate of oxygen uptake was determined polarographically at 20°C with a Clark-type (Rank Brothers) oxygen electrode inserted in a cuvette on a magnetic stirrer. Respiration rates were calculated on the basis of 265 μM oxygen in the air-saturated reaction medium at 20°C and were expressed in nmoles O_2 h^{-1} mg d.w.$^{-1}$.

2.6 Isolation of mitochondria and oxygen uptake determinations

The procedures for harvesting the sporidia and for obtaining mitochondria described by White and Thorn[8] were adapted with minor modifications. To obtain sporidia the fungus was grown in 2-litre Erlenmeyer flasks

containing 500 ml of the liquid medium. An actively growing sporidial suspension (50 ml) was used to inoculate each flask. After a 48-h incubation period on a rotary shaker (120 rev. min^{-1} at 20°C) the sporidia were harvested by centrifuging at 3000*g* for 10 min and washed with phosphate buffer (0·05 M, pH 7·0) containing EDTA (10 mM), sucrose (0·25 M) and bovine serum albumin (BSA; 1·5 g $litre^{-1}$). The harvested sporidia were divided into batches of 7–10 g and kept at −20°C for 24 h. These frozen sporidial pellets were ground for 7 min in a mortar after addition of twice the weight of acid-washed sand and one part of the ice-cold buffer. Mycelial debris and the sand were removed by centrifuging at 3000*g* for 5 min. The supernatant was centrifuged at 21 000*g* for 10 min to yield the mitochondrial pellet. Mitochondria were suspended by means of a glass homogeniser in the homogenisation buffer without BSA to a protein concentration of 1·5–2·0 mg ml^{-1}. All steps to obtain mitochondria preparations were carried out at 4°C. Oxygen consumption was measured at 20°C with 1·9 ml reaction mixture and 0·1 ml mitochondria (usually 150–200 μg protein). The reaction mixture contained phosphate buffer (0·05 M; pH 7·2), sucrose (0·25 M), EDTA (5 mM), magnesium chloride (5 mM), potassium chloride (0·01 M), BSA (1 g $litre^{-1}$), cytochrome *c* (10 μM) and ADP (1·5 mM). NADH (1·5 mM) was used as the respiratory substrate. The reaction was started by the addition of mitochondria and the total amount of organic solvent was no more than 10 ml $litre^{-1}$ in the reaction mixture. Cytochrome oxidase and alternative oxidase activities were measured in the presence of SHAM (2 mM) and potassium cyanide (1 mM) respectively. Respiration rates were calculated on the basis of 265 μM oxygen in the air-saturated buffer at 20°C and were expressed in nmoles O_2 min^{-1} mg $protein^{-1}$. The protein content of mitochondrial preparations was determined by the Bio-Rad assay procedure using BSA as the standard.[9]

3 RESULTS

3.1 Fungitoxicity of ICIA 5504 to the wild-type and to the mutant strain

In the absence of fungicide all the sporidia of both wild-type and mutant strains germinated. Sporidial germination was highly sensitive to ICIA 5504 in the wild-type strain (Fig. 2). A dose-dependent decrease in the ratio of germinated sporidia was observed. The ED_{50} value, the concentration causing 50% inhibition of sporidial germination and the MIC (minimal inhibitory concentration) value were approximately 0·025 and 0·1 μg ml^{-1} respectively. In contrast, a dose-dependent decrease of sporidial germination was observed in the mutant strain (K1775) only at low fungicide concentrations (up to 0·5 μg ml^{-1}). A plateau in the ratio of germinated sporidia at the level of 40% was observed at high concentrations.

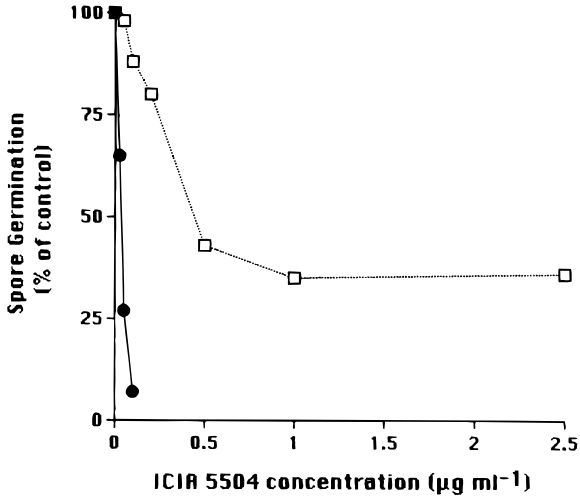

Fig. 2. Inhibition of spore germination by ICIA 5504 in (●) wild-type and (□) mutant strain of *Septoria tritici*. All spores ($n = 120$) of both strains germinated in the absence of inhibitor.

Results of growth sensitivity tests in liquid media with these isolates are shown in Fig. 3. The growth of wild-type was found to be even more sensitive than sporidial germination. The ED_{50} and MIC values were 0·01 and 0·05 μg ml^{-1} respectively. The sensitivity of the mutant strain to ICIA 5504 was reduced approximately 10 times at either the ED_{50} or the MIC values. A dose-dependent decrease of growth was observed for both strains. Some residual growth of the mutant strain occurred at 1 μg ml^{-1} of ICIA 5504.

3.2 Fungitoxicity of ICIA 5504 on wheat

Assessment of the disease on wheat plants showed that the wild-type (K1097) and the mutant (K1775) strains

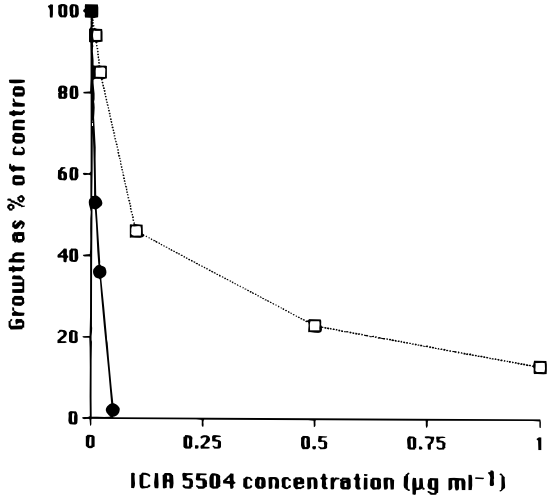

Fig. 3. Effect of ICIA 5504 on growth of (●) wild-type and (□) mutant strain of *Septoria tritici* in liquid medium. The growth of untreated cultures at 48 h (measured as OD^{48}_{450}-$OD^{0}_{450}/OD^{0}_{450}$) was approximately 11 for both strains.

were both pathogenic (65 and 62% sporidial germination and 88 and 59% disease cover respectively) under the test conditions. At the three lower concentrations tested (0·03, 0·1 and 0·3 μg ml^{-1}) a dose-dependent control of *S. tritici* was seen (Fig. 4), and at the higher concentrations of spray the disease control was complete. Surprisingly, the mutant strain K1775 was more susceptible to ICIA 5504 than the wild-type. The experiment has been repeated with similar results (data not shown).

3.3 Inhibitor sensitivity of glucose oxidation by sporidia from the wild-type and the mutant strains

Measurements of glucose oxidation by whole cells revealed a very striking difference between the methoxyacrylate-resistant mutant and the wild-type (Fig. 5). With the wild-type, a dose-dependent decrease of substrate oxidation was observed up to 0·1 μg ml^{-1} ICIA 5504, which caused 80% inhibition of oxygen uptake. However, the inhibition of respiration was not complete and the residual oxygen uptake rate (20% of control) remained uninhibited over a further 10-fold increase of inhibitor concentration. In contrast, glucose oxidation was stimulated by ICIA 5504 up to 150% in the mutant sporidia. The respiration of the wild-type strain was strongly inhibited by antimycin A or cyanide (81% inhibition at 5 μg ml^{-1} antimycin A or 1 mM potassium cyanide), whereas that of the mutant was not inhibited or sometimes stimulated.

These observations suggest the presence in the mutant isolate of an efficient alternative respiratory system which is not inhibited by antimycin A, cyanide or ICIA 5504. Because hydroxamates are known specific inhibitors of cyanide-resistant respiration in

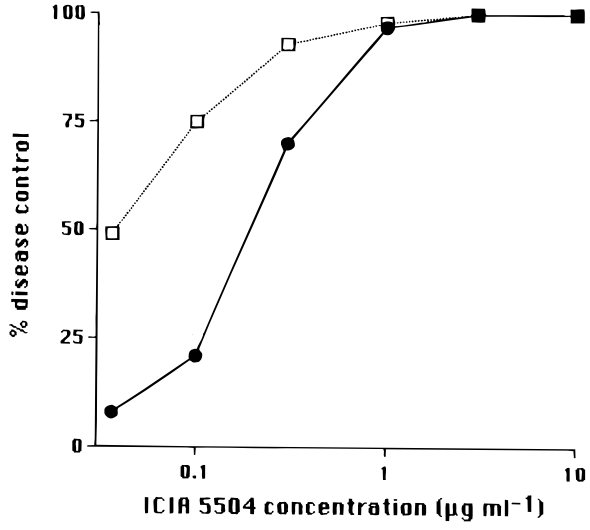

Fig. 4. Control of (●) wild-type and (□) mutant strain of *Septoria tritici* on wheat by ICIA 5504 (2-h protectant test). Untreated plants had 88% (wild type) and 59% (mutant) disease cover at 28 days after treatment.

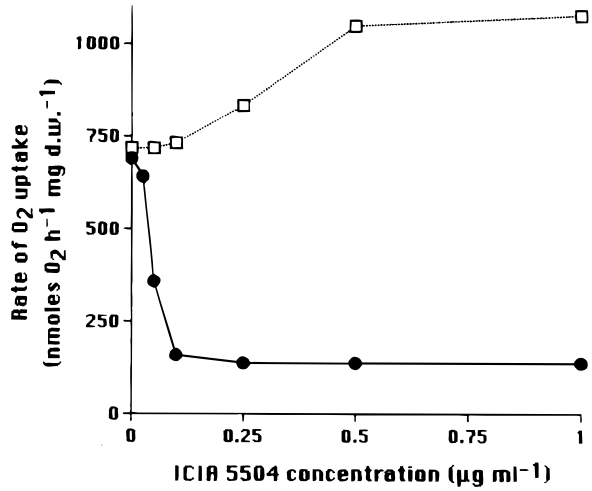

Fig. 5. Effect of various concentrations of ICIA 5504 on oxygen uptake by (●) wild-type and (□) mutant sporidia of *Septoria tritici* utilizing glucose as substrate, after 1 h preincubation.

various organisms,[10,11] the effect of SHAM on substrate oxidation was studied. Dose-response experiments (Fig. 6) in mutant sporidia, showed that, in the absence of cyanide, the rate of glucose oxidation was not affected by SHAM. This indicates that oxygen uptake under control conditions is mediated only by the cytochrome pathway. In the presence of cyanide in the reaction mixture, a strong inhibition of respiration was observed with 2 mM SHAM (Fig. 6).

The addition of SHAM (2 mM) to the reaction mixture resulted in a dose-dependent inhibition of respiration rate by ICIA 5504 in both strains (Fig. 7). Only a slight difference in the sensitivity to fungicide between wild-type and mutant strains was observed and the oxygen uptake was completely abolished at 0·5 and 1·0 μg ml^{-1} of ICIA 5504, respectively.

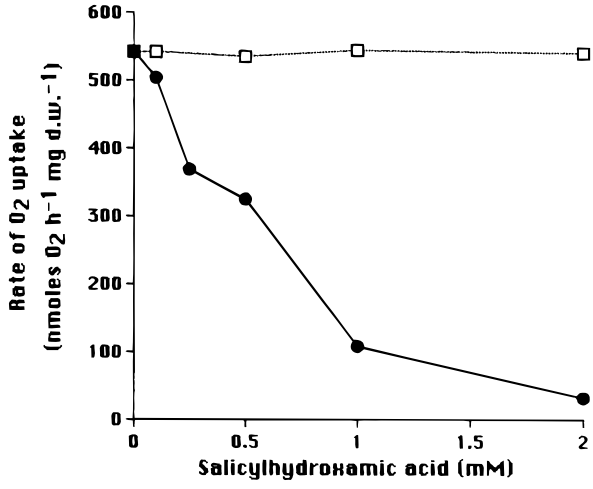

Fig. 6. Effect of various concentrations of salicylhydroxamic acid (SHAM) on oxygen uptake (glucose substrate) by mutant sporidia of *Septoria tritici* in (●) presence and (□) absence of cyanide (1 mM).

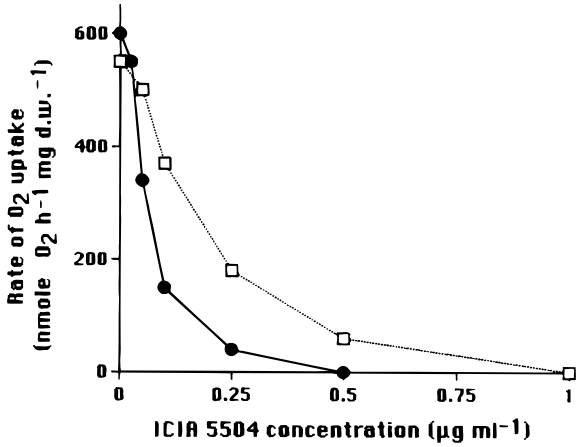

Fig. 7. Effect of various concentrations of ICIA 5504 on oxygen uptake (glucose substrate) by (●) wild-type and (□) mutant sporidia of *Septoria tritici* in the presence of (2 mM) SHAM.

3.4 Inhibitor sensitivity of NADH oxidation by mitochondria from wild-type and mutant sporidia

The behaviour of mitochondria from the wild-type and the mutant cells is shown in Table 1. Antimycin A (5 μg ml^{-1}) and ICIA 5504 (1 μg ml^{-1}), both inhibitors of cytochrome bc_1 complex at the Q_i and Q_o sites respectively,[6] did not completely inhibit NADH oxidation in either strain, but were more effective against the wild-type.

SHAM (2 mM) induced a slight inhibition of mitochondrial respiration in the mutant whereas it had no effect towards the sensitive strain. Cyanide (1 mM) completely inhibited substrate oxidation in the wild-type strain, but a small amount of residual respiration in the mutant remained. In the sensitive strain the addition of SHAM to antimycin A or to ICIA 5504 gave no additional effect, whereas, in the resistant mutant, SHAM slightly increased the level of inhibition, indicating a small electron flow through the alternative pathway. For complete inhibition of antimycin A- and methoxyacrylate-insensitive respiration, addition of SHAM and cyanide was required in the mutant mitochondria. Since the reaction mixture contained cytochrome *c*, *S. tritici* mitochondria appear to possess an antimycin A- and methoxyacrylate-insensitive NADH-cytochrome *c* reductase, as in the case with mitochondria from other sources.[12] Because there is not a specific inhibitor of the above electron transfer, the assay of the methoxyacrylate sensitivity of electron transfer through the cytochrome bc_1 complex is possible after inhibition of alternative oxidase by hydroxamates and subtraction of electron flow through NADH-cytochrome *c* reductase from the recorded rates of exogenous NADH oxidation. The results of such measurements (Table 2) show that ICIA 5504 is equally inhibitory to both type of mitochondria and a complete inhibition of NADH oxidation through the cytochrome bc_1 complex occurs at a concentration of 0·5 μg ml^{-1}.

TABLE 1
Rates of NADH Oxidation by Mitochondria from Wild-Type (K1097) and Mutant (K1775) Sporidia of *Septoria tritici* as Affected by ICIA 5504 and Other Known Inhibitors of Electron Transport

	Rate of O_2 uptake (nmol O_2 min^{-1} mg protein^{-1})	
Treatment	*K1097*	*K1775*
Control	322	330
Antimycin A	74	111
ICIA 5504	71	102
Potassium cyanide	0	22
SHAM	320	305
Antimycin A + SHAM	73	87
ICIA 5504 + SHAM	70	76
Antimycin A + SHAM + potassium cyanide	0	0
ICIA 5504 + SHAM + potassium cyanide	0	0

Concentrations of inhibitors: Antimycin A, 5 μg ml^{-1}; ICIA 5504, 1 μg ml^{-1}; potassium cyanide, 1 mM; SHAM, 2 mM.

TABLE 2
Effect of ICIA 5504 Concentration on NADH Oxidation *via* Cytochrome Complex bc_1 of Wild-Type (K1097) and Mutant (K1775) Mitochondria of *Septoria tritici*

	O_2 consumption (nmol O_2 min^{-1} mg protein^{-1})[a]	
ICIA 5504 (μg ml^{-1})	*K1097*	*K1775*
0	248	243
0·01	184	160
0·1	45	32
0·5	0	0

[a] The rate of SHAM- and antimycin A-insensitive portion of O_2 consumption was subtracted from the recorded rates of exogenous NADH oxidation.

4 DISCUSSION

When a new type of fungicide is introduced into major agricultural outlets, the risk of resistance has to be assessed, and there are guidelines available from currently used fungicides indicating the important factors which need consideration.[13] Until resistance occurs in the field, laboratory studies are only an indicator and are necessarily limited in scope, as well as interpretation. This work is part of a programme aimed at understanding what might be the situation with ICIA 5504. Other studies attempting to raise strains of *Neurospora crassa* Shear & Dodge resistant to ICIA 5504 for

biochemical and genetic analysis have produced strains with low levels of resistance (Grindle, M. & Heaney, S.P., pers. comm.).

The *S. tritici* mutant strain K1775, with a resistant factor of about 10 to ICIA 5504 in liquid culture, was the only resistant plant pathogenic isolate available when the present work was initiated. This mutant, selected with ICIA 5504, develops high rates of cyanide-, antimycin A- and methoxyacrylate-insensitive substrate oxidation. Study of whole cell respiration revealed that alternative respiration was also present in the wild-type strain, accounting for the incomplete inhibition of oxygen uptake by ICIA 5504. In mutant sporidia, instead of inhibition, there was a stimulated respiration in the presence of methoxyacrylate compound due to the shift of electron transfer to the alternative oxidase.

According to the view receiving much support over a number of years, ubiquinone is the branching point between the cytochrome and the alternative pathway.[12,14] Thus, respiration is uninhibited by inhibitors of cytochrome complexes III and IV in the organisms with an active alternative pathway. The mechanism of regulation of alternative oxidase is still not clear and there is little information regarding the nature of the enzyme. However the current information supports the view of the alternative oxidase as a simple enzyme composed of few proteins.[15,16] From the first studies on the genetic basis of the alternative pathway in *Neurospora crassa*, it was concluded that the alternative oxidase activity is encoded by structural and regulatory genes.[17] Later molecular studies using alternative oxidase antibody confirmed the above conclusion.[18] No studies of the alternative oxidase of *Septoria* species have been undertaken, so the question of the structural or regulatory gene mutation involved in the *S. tritici* mutant remains to be answered.

The glasshouse test, however, shows that, despite the same fitness of the mutant in terms of germination, rate of growth and substrate oxidation, and in its pathogenicity in the protectant test, the alternative respiration did not alter the susceptibility of the resistant isolate to ICIA 5504 *in vivo*. Obviously the limited amount of energy which is provided by the alternative respiration (methoxyacrylate-insensitive) in the mutant *S. tritici* strain in the presence of ICIA 5504 is inadequate for efficient parasitic growth on wheat. ATP can only be synthesised at one site as electrons flow through complex I (NADH: ubiquinone reductase) of the cytochrome pathway[16] when oxygen uptake is mediated by the alternative pathway.

Our study does indicate that modulation of alternative oxidase can be one mechanism of resistance to fungicidal respiratory inhibitors acting at the Q_o site of cytochrome bc_1 complex, but, as described above, the presence of the alternative oxidase does not affect the practical outcome in the in-vivo situation. Under some circumstances an alternative pathway of respiration is present in sensitive strains of fungi, as has been described in *Pyricularia oryzae* Cavara to the compound SSF-126, which acts at the same site in the cytochrome bc_1 complex.[19,20]

Yeast mutants resistant to antibiotics which inhibit the cytochrome bc_1 complex are well characterised, and the mutations in the mitochondrial cytochrome *b* gene mapped.[21] There is, however, little indication of how frequently they occur spontaneously, as they have only been raised using special techniques. It is not possible to extrapolate from them what will happen in a plant pathogenic fungus under field conditions. It is clear that this resistant strain of *S. tritici* has a cytochrome *b* which is as sensitive to ICIA 5504 (Table 2) as the sensitive parent, so it does not carry a cytochrome *b* mutation.

ACKNOWLEDGEMENTS

This work was done when Dr. B. N. Ziogas was a Visiting Fellow in the Exploratory Plant Sciences Department of Zeneca Agrochemicals, Jealott's Hill Research Station. The authors thank their colleagues in the Bioscience and Plant Pathology Section, especially S. P. Heaney, for their valuable assistance.

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